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Full Length Research Paper

Isolation of an ascorbate peroxidase in *Brassica napus* and analysis of its specific interaction with ATP6

Zhi-bin Liu, Yu-fei Yue, Jun-bei Xiang, Jian-mei Wang, Jun Wu, Xu-feng Li and Yi Yang*

Key Laboratory of Bio-resources and Eco-environment, Ministry of Education, College of Life Science, Sichuan University, Chengdu, Sichuan 610064, P.R. China.

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In *Brassica napus*, ATP6 is related to *Pol* cytoplasmic male sterility. To understand the mechanism of *Pol* CMS, proteins which interact with ATP6 were screened in a yeast two-hybrid system. A partial sequence of a putative and ascorbate peroxidase (*Bn-APX*) was isolated from *Brassica napus*. By use of rapid amplification of cDNA ends method, the full length of *Bn-APX* coding sequence was cloned. The deduced amino acid sequence contained 438 amino acid residues with a conserved ascorbate peroxidase domain and shared 77% identity with that of *APX* from *Arabidopsis thaliana*. Further analyses revealed that the region of *Bn-APX* interacting with ATP6 was at its c-terminal. It was also observed that the expressions of *ATP6* and *Bn-APX* were strongly similar in the floral tissue of CMS line and the restoring line. Furthermore, in restoring line, the expression of *Bn-APX* is higher in the flower than that in other tissues.

Key words: *ATP6*, *Pol* CMS, yeast two-hybrid system, ascorbate peroxidase.

INTRODUCTION

Cytoplasmic male sterility (CMS), a maternally inherited condition in which a plant is unable to produce functional pollen, has been observed in numerous species (Laser and Lersten, 1972). Detailed molecular analyses of a number of CMS systems in various plant species have revealed that male sterility is associated with some essential mitochondrial genes, accompanied by either appearance of novel, often chimerical transcripts or altered expression usually in a tissue-specific manner (Schnable and Wise, 1998; Hanson and Bentolila, 2004). Such changes in mitochondrial gene expression usually result from rare mitochondrial genome mutations, alien mitochondrial substitutions subsequent to wide hybridization or mitochondrial recombination in somatic hybrids. Although the regions of the mitochondrial genome relative to certain forms of CMS have been identified, the molecular basis of the trait is not precisely understood in any system.

Brassica napus, which is widely grown as the oilseed crop of rape or canola, offers several advantages as a system for the molecular analysis of CMS. The relatively simple organization of its mitochondrial genome in the *Brassica* and allied genera (Palmer and Herbon, 1986) facilitates detailed analysis of structural differences between sterile and fertile mitochondrial DNA (Makaroff and Palmer, 1988). In addition, the capability of producing *Brassica* somatic hybrids with recombinant mitochondrial genomes potentially allows for direct genetic analysis of the cytoplasmic determinants of CMS. There is also considerable interest in applying *Brassica* CMS in the production of hybrid rapeseed, because seed yield in *Brassica napus* hybrids may be enhanced by as much as

*Corresponding author. E-mail: yangyi528@scu.edu.cn. Tel: 86-28-85412281. Fax: 86-28-85410957.

Abbreviations: CMS, Cytoplasmic male sterility; **RT-PCR,** reverse transcriptase PCR; **SMART,** switching mechanism at 5' end of RNA transcript; **SD,** synthetic dropout; **GAL,** galactose; **GAL4BD,** GAL4 binding domain; **GAL4AD,** GAL4 activation domain; **APX,** ascorbate peroxidase; **Bn-APX,** *Brassica napus* ascorbate peroxidase; **HMG,** high mobility group; **ROS,** reactive oxygen species; **RACE**, rapid amplification of cDNA ends; **ONPG,** Ortho nitro phenyl β-D-galactopyranoside.

60% above that of parental lines.

Polima cytoplasm confers a relatively temperature sensitive male sterility (Polowick and Sawhney, 1988; Young et al., 2004) and because of the availability of restorer genotypes, this system appears to be advent-ageous for hybrid rapeseed production. The *ATP6* mitochondrial gene region has also been found to be associated with the *Polima* or *Pol* CMS system of *B. napus* (Singh and Brown, 1991; Witt et al., 1991; Handa and Nakajima, 1992). In this case, the *ATP6* gene is situated downstream of and co-transcribed with a chimerical mitochondrial gene open reading frame named *orf224* (Singh and Brown, 1991). The N-terminal coding and 5' non-coding regions of this chimerical gene are derived from a normal mitochondrial gene, *orfB*, while the origin of the remaining portion is unknown.

In order to study the reason that an alternation of *ATP6* leads to *Pol* CMS, we screened ATP6 interacting proteins in a yeast two-hybrid system and identified Bn-APX, a putative ascorbate peroxidase. The interaction between ATP6 and *Bn-APX* was then analyzed. We also analyzed the expression of *ATP6* and *Bn-APX* in the flower of the CMS line and the restoring line of *Brassica napus.*

MATERIALS AND METHODS

Plant materials

The *Pol* CMS and restorer genotype of the *B. napus* lines used in this study have been described previously (Singh and Brown, 1991). *Pol* CMS and the fertility restorer lines were grown under normal growth conditions (day/night temperatures 22/16℃, 16 h photoperiod), with a relative humidity about 70%. Flower, leaf and stem were collected and stored at - 80℃ until use.

RNA isolation and reverse transcription

Total RNA was prepared from the fresh leaf using the RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA solutions were quantified by spectro-photometer and the quality was verified by gel electrophoresis. Reverse transcription was performed according to the Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, China) and the harvested complementary cDNA was deposited at -20℃ until used.

Construction of Clones

The Matchmaker Two-Hybrid System (CLONTECH, Palo Alto, CA, USA) was used to detect the physical interaction between ATP6 and its interacting protein. The entire coding sequences of *ATP6* (accessionnumber S47089) were amplified by PCR using primers (atp1: 5'-CGGGATCCCTA, TGAATCAAATAGGGCTGGT-3' / atp2: 5'-AACTGCAGAATGGAGATTTATAGCATCATTC-3') and cloned into *Pst* I and *BamH* I sites in pGBKT7. *Bn-APX* and truncated versions of *Bn-APX* (APX_{1-140}, APX_{1-250}, $APX_{140-439}$, $APX_{250-439}$ and $APX_{140-250}$) were constructed into the *EcoR* I and *BamH* I sites of pGADT7 by PCR amplification. All plasmids were sequenced for correct construction.

Yeast two-hybrid screening

The *B. napus* cDNA library was constructed using RNA from floral tissue of fertility restorer lines□□messenger RNA transcripts are efficiently copied into ds cDNA using BD SMART (Switching Mechanism at 5' end of RNA Transcript) technology and cDNA library construction was performed as described (Zhu et al., 2001). The pGBKT7-ATP6 and library plasmids were co-transformed into yeast host strain AH109. A total of 1×10^8 transformants were plated onto synthetic dropout (SD) selection medium that lacked tryptophan, leucine and histidine. Yeast transformants that appeared on the selection medium within 5d were streaked on a nylon membrane soaked with SD medium lacking tryptophan, leucine and histidine and then filter-lift assays were performed as described in the protocol supplied by the manufacturer. The pGADT7 plasmids isolated from both his3- and lacZ- positive yeast transformants were co-transformed with the empty pGBKT7 into the AH109 yeast strain to determine the specificity of growth.

5'RACE and full-length cDNA cloning of *Bn-APX*

The 5'RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA), according to the instructions of the manufacturer. By comparing and aligning the sequence of yeast two-hybrid and 5'RACE products, the full-length cDNA sequence of *Bn-APX* was obtain, which then was amplified via PCR using a pair of primers (apx1: 5'-AGATGGCTCTATCT CTCTCCGCC-3'/apx2: 5'-TTAGAAACCAGAGTAATCAGAGGA-3') and then sequenced.

β-Galactosidase activity assay

Various pairs of the pGBKT7 and pGADT7 plasmids were co-transformed into yeast reporter strain AH109. Transformants were plated onto SD medium lacking tryptophan and leucine and incubated for 5 days at 30℃. The colonies were collected and assayed for β-galactosidase activity. β-Galactosidase assays with ONPG and X-gal as substrates, respectively, were performed, as described previously (Matchmaker two-hybrid system, Clontech).

Expression analysis of *ATP6* and *Bn-APX* by quantitative RT-PCR

Real-time PCR was performed using the LightCycler Quick System 350S (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq (Takara, China). Each PCR reaction contained 1X SYBR Premix Ex Taq, 0.2 μ M of each primer and 2 μl of a 1:5 dilution of the cDNA in a final volume of 20 μl. The following PCR program was used: initial denaturation, 95℃, 60 s; PCR, 40 cycles of 95℃, 10 s, 57℃, 15s, and 72℃, 15s. In melting curve analysis, PCR reactions were denatured at 95℃, reannealed at 55℃, then a monitored release of intercalator from PCR products or primer dimmers by an increase to 95℃ with a temperature transition rate of 0.1℃ s^{-1}. To create a standard curve, homologous standards for each gene were used as external standards in all experiments. cDNA quantities were calculated by the second derivative maximum methods of Light-Cycler Software Ver.3.5 (Roche Diagnostics, Mannheim, Germany) and all quantifications were normalized using β-actin mRNA as an internal control. The primers were used as follow: β-actin: 5'-GTGGGGATGGAAGCTCCTG-3' and 5'-GTGATCTCTTTGCTCATACGGTC-3'; *ATP6* 5'-AGGAGTCCC ACTGCCGTTAG-3' and 5'-CATACATAGCATAGTCCAAGCGAAC-

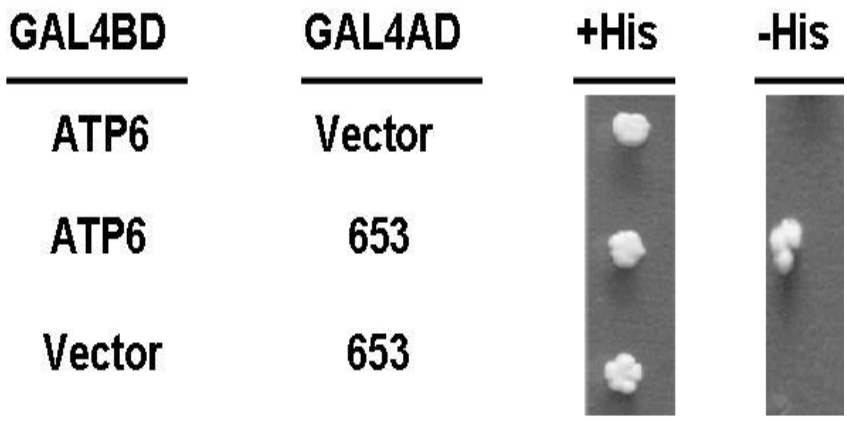

Figure 1. The interaction between *ATP6* and the designated 653 protein in the yeast two-hybrid system. The proteins fused to the GAL4 binding domain (GAL4BD) or GAL4 activation domain (GAL4AD) were indicated at the left panel. Empty vectors for negative controls of each assay were represented as vector. In the right panel, the growth of yeast cotransformants containing both GAL4BD and GAL4AD fusion proteins was measured on medium with histidine or without histidine.

3'; *APX* 5'-AGTTCGTAGCTGCTAAATATTCCAC-3' and 5'-GGA GTTGTTATTACCAAGAAAGTG-3'.

RESULTS

Isolation of proteins that interact with ATP6

To isolate proteins that interact with ATP6, the entire ATP6 protein that fused with GAL4 binding domain (GAL4BD) was first used as bait and screened in a yeast two-hybrid system. In the yeast strain AH109, ATP6 did not activate the transcription of reporter genes. pGBKT7-ATP6 and pGADT7 inserted by cDNAs were co-transformed and screened by growth selection on medium lacking histidine and by the activity of β-galactosidase. A clone designated 653 was isolated as a clone that activated transcription of the two reporter genes in the presence of ATP6. The specific interaction was confirmed by the re-introduction of plasmid extracted into yeast AH109 (Figure 1).

cDNA cloning of full-length Bn-APX

Sequence analysis revealed that clone designated 653 encoded the partial sequence of a putative ascorbate peroxidase (APX) fused in-frame with GAL4AD. The 5'RACE was performed to get the full-length *Bn-APX*. An 831 bp fragment of *Bn-APX* was obtained by yeast two-hybrid. 5'RACE yielded a single 720 bp fragment, This product overlaps the original 831 bp sequence by 103 bp. Assembly of the yeast two-hybrid and 5'RACE product sequences yielded a putative full-length of *B. napus* ascorbate peroxidase (Bn-APX) open reading frame consisting of 1317 bp (GenBank accession FJ965556), which encodes a polypeptide of 438 amino acids. Comparison of the amino acid sequence of *Bn-APX* with homologs of *Brassica oleracea* and *Arabidopsis thaliana* revealed 95 and 77% identity, respectively (Figure 2). A NCBI Conserved Domain Search revealed that the cloned *Bn-APX* sequence encodes a protein belonging to the ascorbate peroxidase family.

Characterization of specific interaction of ATP6 and Bn-APX

Since clone designated 653 encoded a partial sequence of *Bn-APX*, the region of *Bn-APX* that is required for interaction with the ATP6 protein was further characterized. The entire and truncated versions of *Bn-APX* (1-140, 1-250, 140-439, 250-439 and 140-250) were cloned into pGADT7 to fuse to the GAL4 activation domain (GAL 4AD) and used for the determination of interaction. Moreover, the clones containing both pGBKT7-ATP6 and pGADT7 ligated entire or truncated versions of Bn-APX, grew on the SD-Leu-Trp-His-Ade medium and were verified by PCR. The results showed that *Bn-APX* $_{140\text{-}439aa}$, *Bn-APX* $_{250\text{-}439aa}$ and *Bn-APX* $_{1\text{-}439aa}$ could grow on SD medium lacking tryptophan, leucine and histidine. Furthermore, they became blue using X-gal as a substrate (Figure 3). Moreover, β-galactosidase activity was assayed in liquid using ONPG as a substrate. The whole region of *Bn-APX* contributed to the interaction and the Bn-APX$_{250\text{-}439}$ showed a higher reporter activity when

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Brassica napus     MALSLSAASHSLCSSSTTRVSLPPAAVSSSSSSPSSPSLVSFSSLRSLAS   50
Brassica olerace   MALSLSAASHSLCSSSTTRVSLPPAAVSSSSSSPSSPSLVSFSSLRSLAS   50
Arabidopsis        MSVSLSAASHLLCSSS..TRVSLSPAVTSSSSSP.....VVALSSSTSPHS   43

Brassica napus     SSSSSSSSSSLFPHSPSLVCRKHPNRGSSNTVVSPTRAAASDAAGLKSAKE  100
Brassica olerace   SSSSSSSSS.LFPHSPSLVCRKHPNRGSSNTVVSPTRAAASDAAGLKSAKE   99
Arabidopsis        LGSVASSS.LFPHSSFVLCKKHPINGTSTRMISP.KCAASDAAGLISAKE   91

Brassica napus     DIKVLLRTKFCHPILVRLGWHDAGTYNKNIEEWPCRGGANGSLRFEPELK  150
Brassica olerace   DIKVLLRTKFCHPILVRLGWHDAGTYNKNIEEWPCRGGANGSLRFEPELK  149
Arabidopsis        DIKVLLRTKFCHPILVRLGWHDAGTYNKNIEEWPLRGGANGSLRFEAELK  141

Brassica napus     HAANAGLVNALKLIEPIKEKYSNISFADLFGLASATAVEEAGGPEIPMKY  200
Brassica olerace   HAANAGLVNALKLIEPVKEKYSNISYADLFGLASATAVEEAGGPEIPMKY  199
Arabidopsis        HAANAGLLNALKLIGPLKDKYPNISYADLFGLASATAIEEAGGPDIPMKY  191

Brassica napus     GRVDVSAPEGCPEEGRLPDAGPPSPADHLREVFYRMGLNDKEIVALSGAH  250
Brassica olerace   GRVDVSAPEGCPEEGRLPDAGPPSPADHLREVFYRMGLNDKEIVALSGAH  249
Arabidopsis        GRVDVVAPEGCPEEGRLPDAGPPSPADHLRDVFYRMGLDDKEIVALSGAH  241

Brassica napus     TLGFSRPDRSGWGKPETKYTKAGPGEPGGGSWTVKWLKFDNSYFKDIKEK  300
Brassica olerace   TLGFSRPDRSGWGKPETKYTKAGPGEPGGGSWTVKWLKFDNSYFKDIKEK  299
Arabidopsis        TLGFARPDRSGWGKPETKYTKTGPGEAGGGSWTVKWLKFDNSYFKDIKEK  291

Brassica napus     RDEDLLVLPTDAALFEDPSFKYYAEKYAGDPAAFFKDYAEAHAKLSNLGA  350
Brassica olerace   RDEDLLVLPTDAALFEDPSFKNYAEKYAGDPAAFFKDYAEAHAKLSNLGA  349
Arabidopsis        RDDDLLVLPTDAALFEDPSFKNYAEKYAEDVAAFFKDYAEAHAKLSNLGA  341

Brassica napus     KFDPPEGIII DNGPAGGEKFVAAKYSTGKKELSDSMKKKIRAEYEAIGGS  400
Brassica olerace   KFDPPEGIII DNGPAGGEKFVAAKYSTGKKELSDSMKKKIRAEYEAIGGS  399
Arabidopsis        KFDPPEGIVIENVP...EKFVAAKYSTGKKELSDSMKKKIRAEYEAIGGS  388

Brassica napus     PDNPLPTNYFLNIIIIAISVLVLLFTFLGNNNSSDYSG              437
Brassica olerace   PDKPLPTNYFLNIIIIAISVLVLLFTFLGNNNSSDYSG              436
Arabidopsis        PDKPLPTNYFLNIIIIAIGVLVLLSTLFGGNNNSDFSG              425
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Figure 2. Alignment of amino acid sequences of putative *Brassica napus* ascorbate peroxidase with *Brassica oleracea* (GenBank accession BAD14931) and *Arabidopsis thaliana* (GenBank accession CAA67426). HMG (High Mobility Group) domain is from 312 to 423.

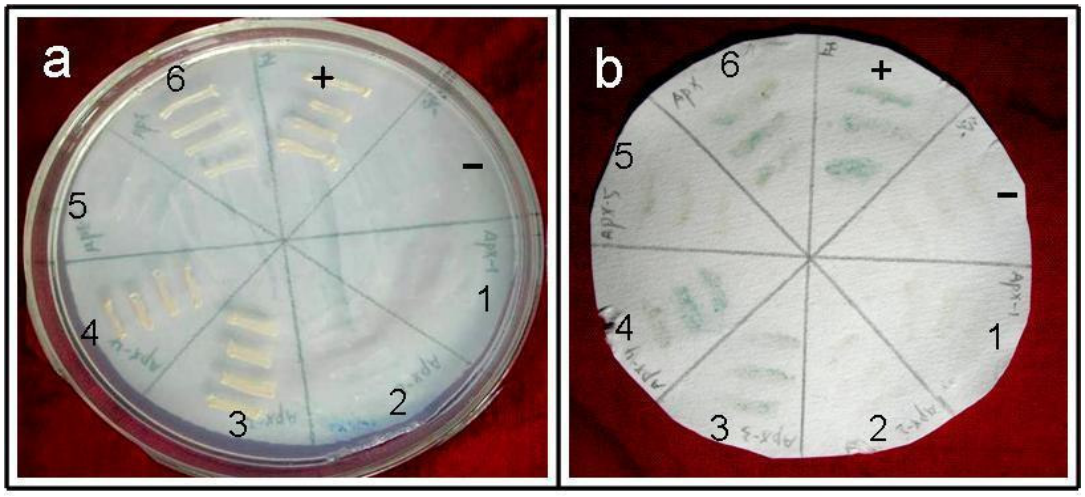

Figure 3. a: The yeast cotransformants containing both GAL4BD and GAL4AD fusion proteins grew on medium without histidine. **b:** β-galactosidase activity of each yeast cotransformant was monitored by blue staining (+: positive control, -: negative control, 1:GAL4AD-APX$_{1\text{-}140}$, 2:GAL4AD-APX$_{1\text{-}250}$, 3:GAL4AD-APX$_{140\text{-}439}$, 4:GAL4AD-APX$_{250\text{-}439}$, 5:GAL4AD-APX$_{1\text{-}140}$, and 6:GAL4AD-APX).

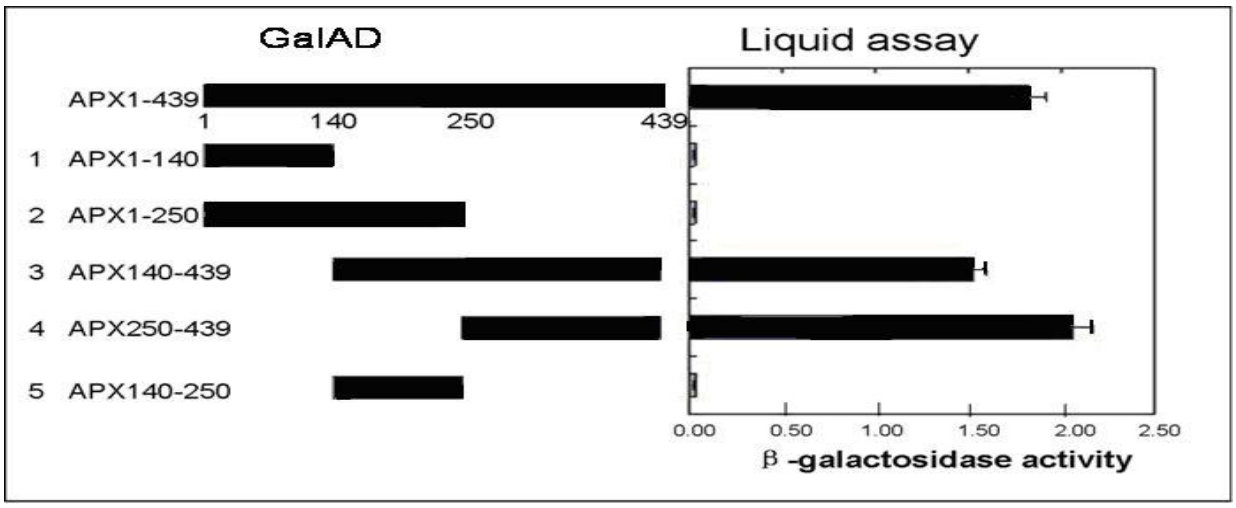

Figure 4. Schematic diagrams of truncated *Bn-APX* and quantitative analysis of the β-galactosidase activity by using ONPG as the substrate. Data were presented as mean ± standard error of triplicate.

interacting with ATP6 (Figure 4).

Expression of *ATP6* and *Bn-APX* in the floral tissue of CMS line and the restoring line

To investigate whether *Bn-APX* and *ATP6* were related to CMS, their expression patterns were analyzed in the floral tissue of CMS line and the restoring line using the quantitative RT-PCR method. The *ATP6* expression level was significantly reduced in the CMS and the *Bn-APX* transcripts in restoring were 2.96 folds than in CMS. We also analyzed their expression in the flower, leaf and stem. The result showed that the transcripts of *ATP6* and *Bn-APX* were highest in the leaf and flower, respectively (Figure 5).

DISSCUSSION

In this work, our results indicated that ATP6 could interact with *Bn-APX* and $APX_{250-439}$ was necessary for the interaction through the yeast two-hybrid system. Protein sequence analysis revealed that $APX_{250-439}$ contain a HMG (high mobility group) domain (http://smart.embl-heidelberg.de/), which appears to play important architectural roles in the assembly of protein complexes in a variety of biological processes (Thomas, 2001).

Quantitative RT-PCR data indicated that the expressions of *ATP6* and *Bn-APX* were strongly similar in flowers of both CMS and restoring line. In addition, the transcripts of *ATP6* and *Bn-APX* were found in all tissues of *B. napus* examined, including leaf, stem and flower, which suggested they are constitutively expressed. Furthermore, in restoring line, the expression of *Bn-APX* was the highest in the flower, suggesting that *Bn-APX* plays an important role in plant development and physiology, especially flower development.

Ascorbate peroxidases, a protein family which use ascorbate as the electron donor for H_2O_2 reduction (Asada, 1992), involved in ascorbate-glutathione cycle, utilize reduced glutathione to regenerate ascorbate (Foyer and Halliwell, 1976), while glutathione is regenerated by glutathione reductase. The ascorbate/glutathione cycle is the most important H_2O_2-detoxifying system in the chloroplast, but it has also been identified in the cytosol, peroxisomes and mitochondria (Jimenez et al., 1997).

It is now widely accepted that most environmental stresses such as high temperature and salt, lead to the accumulation of H_2O_2 (Dat et al., 2000; Mittler, 2002). This accumulation has a number of implications for biological processes within the plant as a whole. H_2O_2, maybe closely related to abscission and pollination, has been proposed as signals for programmed cell death during petal senescence (Robson and Vanlerberghe, 2002). Likewise, APX is considered to play a role as a defense line against ROS-induced programmed cell death in petals and other organs of the plant (Bartoli et al., 1995; Rubinstein, 2000; De Pinto, 2006; Rogers, 2006).

Pol cytoplasm confers a relatively temperature sensitive male sterility (fertile in low temperature and sterile in high temperature). The mechanism is unclear so far. It would suggest a hypothesis based on our work: H_2O_2 accumulates while temperature is increasing. Because the *Bn-APX* interacts with chimerical protein (orf224/atp6 co-transcriptions translated), the ascorbate peroxidases activity are depressed. Then excessive H_2O_2 is not scavenged, as signals for programmed cell death during

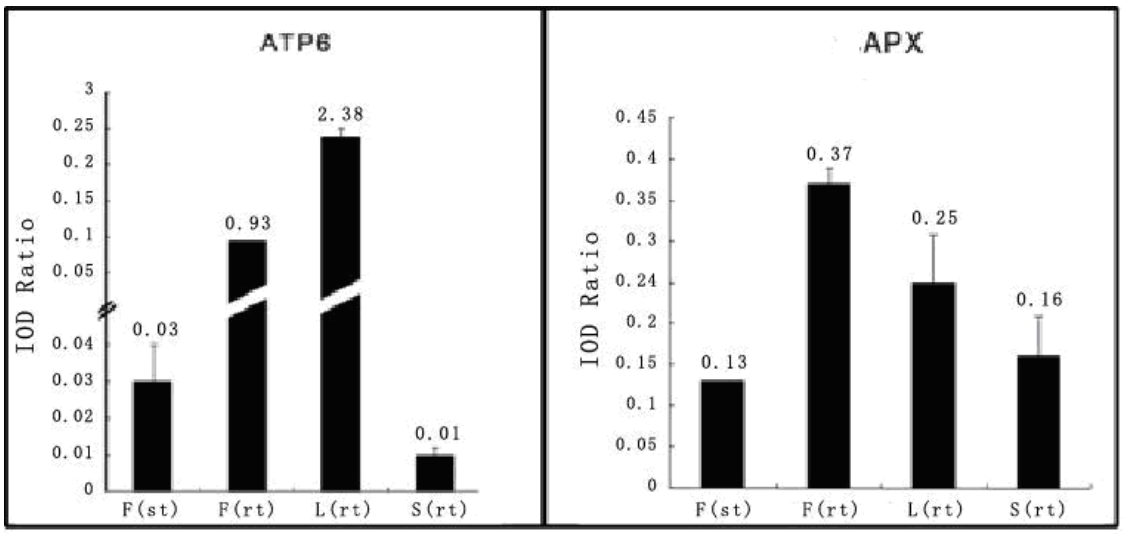

Figure 5. Real-time PCR analysis expression profiles of *Bn-APX* and *ATP6* in different tissues of sterile line and restoring line, respectively. Relative mRNA levels of these genes were normalized with respect to the house-keeping gene β-actin in different tissues of sterile line and restoring line (F: flower, L: leaf, S: stem, st: sterile line, and rt: restoring line). Data were presented as mean ± standard error of triplicate.

petal senescence leading to sterility.

ACKNOWLEDGEMENTS

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